THE FEASIBILITY OF PROPAGATING POLIOVIRUS

IN NON-PRIMATE TISSUES

by

E. ROYLANCE MARTIN

A thesis submitted to the faculty of the University of Utah in partial fulfillment of the requirements for the degree of

MASTER OF ARTS

DEPARTMENT OF BACTERIOLOGY

UNIVERSITY OF UTAH

August, 1956

THIS THESIS FOR THE MASTER OF ARTS DEGREE

by

E. ROYLANCE MARTIN

has been approved by

Chairman, Supervisory Committee

Reader, Supervisory Committee

Reader, Supervisory Committee

Reader, Supervisory Committee

Reader, Supervisory Committee

Head, Major Department

Dean, Graduate School

The author wishes to express his appreciation to Dr. Louis P. Gebhardt and Dr. John G. Bachtold for their guidance and encouragement, and Dr. Paul S. Nicholes, Dr. Stanley Marcus and Mr. Douglas W. Hill for their suggestions and criticism.

TABLE OF CONTENTS

TABLES

Tables:

THE FEASIBILITY OF PROPAGATING POLIOVIRUS IN NON-PRIMATE TISSUES

by

E. ROYLANCE MARTIN

An abstract of a thesis submitted to the faculty of the University of Utah in partial fulfillment of the requirements for the degree of

MASTER OF ARTS

Approved by the faculty committee in

August, 1956

LOUIS P. GEBHARDT, CHAIRMAN, SUPERVISORY COMMITTEE

Department of Bacteriology

University of Utah

August, 1956

Tests concerning the feasibility of propagating poliovirus in non-primate tissues were conducted over a period of two years. The purpose of these tests was to determine whether the cellular specificity of polioviruses was limited to cultures of primate tissues.

Mouse and calf kidney cells were grown in bottles and transferred for several generations. Cells from each new generation were challenged with type II MEF-1 strain poliovirus. The monkey kidney cell system was used for controls.

The use of Melnick's medium containing only 2 per cent calf serum resulted in thin elongation of the calf kidney cells after the sixth generation passage. Increasing the serum content in this medium to 4 per cent aided the growth of the cells. The calf kidney cells grown in Melnick's medium containing 4 per cent calf serum were more rounded yet still elongated after ten generations.

The propagation of poliovirus was not detected in repeated experiments with freshly prepared as well as several subculture generations of either mouse or calf kidney cells by the method employed. It is possible as in the case of Capuchin monkey kidney cells that only a very small number of cells in any given population would be susceptible to the virus. Any occurrence of a very small number of susceptible cells in a given population would result in a failure to observe any cytologic changes. It is possible that small numbers of cells, mouse as well as calf kidney, could have supported the growth of the poliovirus and gone undetected by the tissue culture tube method employed.

THE FEASIBILITY OF PROPAGATING POLIOVIRUS IN NON-PRIMATE TISSUES

INTRODUCTION

Vaccine against poliovirus is at present prepared or cultured only in monkey kidney cells. There has been, in the past, frequent acute shortages of monkeys. Since the shortage of monkeys may, in the future, influence poliovirus vaccine production a search for virus susceptible cells seems justified.

The first and perhaps the most important problem is concerned with the cellular specificity of the viruses of poliomyelitis. The discovery by Enders in 1949 that poliovirus can be propagated in nonneural tissue showed that poliovirus is not strictly a neurotropic virus.

The lack of a susceptible animal tissue more convenient than the primate for the investigation of the various strains of poliovirus provides the stimulus to search for other animal tissues for the propagation of poliovirus in tissue culture.

REVIEW OF LITERATURE

Since the demonstration by Landsteiner and Popper (1), in 1909, that poliomyelitis could be produced in monkeys, various workers have attempted to cultivate the virus *in vitro*.

The general recognition that the virus could be present in the intestinal tract of patients with poliomyelitis and of persons in contact with them emphasized the desirability of further investigation of the possibility of extra-neural multiplication.

The discovery of Enders, Weller and Robbins (2), in 1949, that the virus of poliomyelitis could be propagated in cultures on nonneural tissue, not only made available new techniques for development of a source of virus for immunologic studies, but influenced profoundly and brought evidence to bear on the question of whether the poliomyelitis virus is strictly neurotropic. These investigators were the first to demonstrate that polioviruses, not only propagated in cultures of human and adult nonnervous tissues, but also produced a cytopathogenic change therein. This latter finding made it possible to recognize the presence of virus without the need of resorting to animal inoculation.

Rhodes, *et al*, (3) have confirmed the work of Enders and co-workers and state that the poliovirus multiplies in cultures of human embryonic kidney, post-natal tonsils, and in cultures of monkey testis, lung, kidney, and gut.

Syverton, et al, (4) also reported the successful growth and typing of poliovirus in vitro cultures of He La cells originating from human cancer tissues.

Enders, et al, (5) reported further upon the cytopathogenic effect of the Lansing (Type II) and Brunhilde (Type I) strains of poliovirus on cells which supported their multiplication. This was manifested in suspended cell cultures by the lack of acid production of infected, as compared with uninfected tissues, and by the failure of fragments removed from such cultures inoculated with the virus to exhibit a normal outgrowth of cells when transferred to a plasma medium. These infectious agents were grown in roller-tube cultures of human embryonic skin and muscle tissue and led to destruction of the proliferating cells. This destructive effect associated with a growth of the virus could be inhibited by type specific antiserum. Later experiments with the Leon strain (Type III) revealed this same phenomena. Through the use of these procedures large quantities of fluid were prepared which contained quantities of virus approaching those found in the nervous system of infected animals, but which contained only insignificant amounts of other proteins and tissue derivatives.

These methods have proven to be suitable for the isolation, assay and production of the various strains of polioviruses. These methods also enabled the investigators to demonstrate circulating or humoral antibodies through virus neutralization tests, and have thus proven helpful in attempts to define more clearly the epidemiology of poliomyelitis, and the problem of the immunization of man (5).

Investigators in the field of poliomyelitis appear to be divided between two theoretical approaches to the practical question of immunization of man against this disease. There are those who believe that it may be possible to induce and maintain immunity throughout life with a vaccine consisting of virus in a noninfectious form. Others are of the opinion that lifelong immunity can best be provided through the use of a living attenuated virus for each of the three immunologic types and administered, preferably, by a natural route (6).

Salk (7) reported on the active immunization of man against poliomyelitis using virus, cultured in monkey kidney tissue, as the vaccine source. This vaccine was a mixture of the Mahoney (Type I), MEF 1 (Type II), and Saukett (Type III) strains (the 3 known types) of poliovirus inactivated by treatment with formaldehyde. Salk found that levels of antibody induced by vaccination of man compare favorably with levels which develop after natural infection. This, however, has been refuted by several investigators (8) (9) (10) (11). Sabin, (8) reported on Salk's (7) results with "killed" virus vaccine, given to 16 persons that lived in an institution that had experienced an outbreak of poliomyelitis in 1951, that each person already had antibody titers 1:16 - 1:2,048 before they received the vaccine. These persons exhibited a four fold or greater rise in titer. Sabin further stated that it is well known that it takes very much less antigen to act as a booster than to produce antibody de novo, and concluded that unquestionably the ultimate goal for the prevention of poliomyelitis is immunization with "living" attenuated avirulent virus which will confer immunity for many years or for life. Bodian, (9) stated that there is no doubt that "killed-virus" vaccines are inferior to "live-virus" vaccines as stimulators of antibody production. Cox, (10) emphasized that from a practical standpoint the use of living attenuated viruses as immunizing agents is unquestionably the best method for securing long-lasting and safe protection Preliminary data concerning clinical investigations on attenuated strains of poliovirus reported by Kaprowski, <u>et al</u>, (11) (12), indicates the feasibility of producing a successful "live-virus" vaccine.

Sabin, (8) found that by rapid serial passages of monkey kidney cultures of type I (Mahoney Strain) and type II (YSK Strain) there appeared variants or mutants with markedly diminished virulence for the nervous system of cynomolgus monkeys. Quantitative determinations of various types of activity, of these variants or mutants, have shown that these cultures are mixtures of very large numbers of "nonparalytogenic" particles. This further indicates the feasibility of producing a successful "live-virus" vaccine.

A point of particular interest is that if a vaccine containing nonliving virus is found to be effective, a live-virus vaccine would still be more desirable; and, if such could be developed, it could be used either alone or in conjunction with a killed-virus vaccine to gain a greater immunizing effect (13).

It is understandable that, as a result of the episode of Spring 1955, (14) (15), a prominent doubt developed and still continues to exist concerning the fundamental safety of a killed-virus vaccine. Because of this episode greater precautions have been undertaken to be certain there is no live virus in the vaccine. This requires that tests be applied, not only on samples of the finished material, but on samples removed at several stages during the course of processing. By such a procedure the extent to which a calculated amount of overtreatment has been applied to each batch of vaccine may be demonstrated. This creates what is referred to as a "margin of safety" (16). This may include greater intensity of the inactivation procedure, and result in loss of some of the antigenic capacity of the vaccine. This loss of antigenic capacity would further point to the need of a living attenuated virus vaccine, or to some other form of processed virus, e.g. ultraviolet or gamma-ray irradiation etc.

REVIEW OF LITERATURE CONCERNING WORK ON ANIMAL TISSUES OTHER THAN PRIMATE

The failure of Enders, et al, (17) to obtain any indication of multiplication of poliovirus in suspended cell cultures of tissues from mice and chick embryos suggests that the specificity of the virus for certain cells may be great. These failures not only serve as a kind of control for the results obtained with the tissues of primates, but also afford another demonstration of what would seem to be solid natural resistance on the cellular level. Robbins and Enders conclude that whether or not this resistance can be overcome remains for future study to determine (18).

Smith, et al, (19) tested cell strains (different genera and species sources) that have been thoroughly adapted to in vitro multiplication. Such cells often change their biological characteristics and it appeared desirable to determine whether cells of this sort were susceptible to poliovirus. They found that no multiplication of virus occurred in testicular tissue from mice, hamsters, guinea pigs, rabbits, dogs, and bulls. In the view of poor results obtained with monkey testicular tissue in their flask cultures, they concluded that because of their inability to obtain multiplication with poliovirus in tissue cultures of skeletal and cardiac muscle of monkeys, and testicular tissue of animals other than primate, this is not undeniable evidence for the complete resistance of these tissues, and state that further studies on these and other tissues should be made before a firm decision is reached concerning their susceptibility or resistance to poliovirus.

It is of interest as well as encouraging to observe the results of egg embryo propagation of poliovirus. Kolmer, et al, (20) at the conclusion of their work on egg embryo culture learned that Burnet (21) had also failed to cultivate the virus from monkeys and human spinal cords in egg embryos, and concluded that it would appear definitely established that the virus does not survive or proliferate in egg cultures.

Because of many unsuccessful attempts made to cultivate poliovirus in the developing chick embryo, most investigators were led to believe that this group of agents was not able to parasitize this host and that this characteristic could be used to distinguish poliovirus from other neurotropic agents (22).

Garcia, et al, (22) worked against this idea and in 1952, maintained the MEF-1 strain (type II), after 119 serial passages in suckling hamsters, then for 41 consecutive generations in the developing chick embryo by the yolk sac route of inoculation. They concluded that these results may have been due to increased titers obtained in the serial hamster passages. Cabasso and Cox (23) reported on the cultural and other characteristics of chick-embryo-adapted MEF-1 strain of poliovirus. They concluded that the best source of the virus was from the embryo proper.

It is possible that egg culture of poliovirus may be used on a large scale. It is the opinion of the researchers cited above that more work should be done with tissues other than primate before a firm decision is reached concerning the apparant resistance of non-primate tissues to the poliovirus.

Melnick, et al, (24), in 1943, demonstrated that the South American ringtail monkey (Cebus capucina) has a limited susceptibility to poliovirus in vivo.

Recent experimental work carried out by Kaplan (25) showed that the type II YSK strain of poliovirus, highly cytopathic for rhesus monkey kidney cultures, was without demonstrable effect in capuchin monkey kidney cell cultures.

Kaplan and Melnick (26), in 1955, made the following further observation: Cultures of trypsinized kidneys of South American capuchin monkeys supported the multiplication of type I polioviruses, even though no cytopathic changes were observed by microscopic examination of the cultures. This failure to observe cytologic changes was found to be due to the small proportion of cells in the culture capable of supporting the growth of virus. They found that only an average of 60 cells in a population of 10^5 cells was infected. These viruses remained virulent for the monkey brain and spinal cord after 4 to 5 tissue culture passages. At present there is no evidence for the propagation of type II and III strains in the capuchin cultures. Therefore, it would seem definite that kidney cells of the capuchin monkey do not support growth of type I poliovirus to a significant degree and types II and III are completely insusceptible. This shows a definate variance in a given cell colony and makes way for, or gives impetus to, the search for tissues of other animals that may demonstrate this same variance.

Immunological studies have been carried out by Hammon (27) and others (28) (29) in which "neutralizing factors" or "protective substances" have been found in sera of various animals. The status of these "protective substances" has been uncertain for they were demonstrable only in low dilution and against one strain (Lansing type II), the only strain readily available in most laboratories. Whether one is here dealing with a non-specific neutralizing substance or a specific antibody has been a critical point (30). Identification of these substances as specific antibodies to viruses of poliomyelitis according to Bartell and Klein (30), in 1955, would point strongly to the existence of either an extra-human reservoir of viruses of poliomyelitis or a serologically related group of viruses. The sera of cows and steers were shown by Bartell and Klein (30) to possess neutralizing substances in high titer against poliovirus. These have been shown to be specific antibodies. Calves show a low titer or none at all. Attempts to isolate a virus from the bovine species that might explain the origin of these antibodies have been unsuccessful. Although the antigenic substance giving rise to antibodies against the viruses of poliomyelitis remains unknown, the presence of antibodies in the sera of cows has added stimulus to the search for tissues other than primate for the propagation of poliovirus. Neutralizing antibodies have also been found in the blood of 2% apparently normal monkeys and demonstrated in serum neutralization tests by Miller and Wenner (31).

It may be concluded that from the information available in the literature, further studies on tissues other than primate should be made before a firm decision is reached concerning their susceptibility or resistance to the viruses of poliomyelitis. Different approaches to increase cell susceptibility, such as treatment with cortisone, ultraviolet light, X-irradiation, etc., that may alter the cells, in tissue culture, to allow virus invasion, should be used.

MATERIALS AND METHODS

TISSUES

Mouse kidneys and testes were obtained from young, 21 - 25 day old white mice. Calf kidneys and serum were obtained from the Mc Farlands Meat Packing Company of Salt Lake City, Utah. The tissues were removed as aseptically as possible after the animals were sacrificed and the cell extraction was started within one hour after removal.

VIRUS

Poliovirus type II MEF-1 strain was obtained from Dr. J. G. Bachtold of the University of Utah Poliomyelitis Research Laboratory as monkey kidney cell passage virus. This virus was kept in the deep freezer and thawed just before use.

CHEMICALS

Tissue nutrients medium 199 and Hanks solution, when available, were obtained from the stock solutions used by the University of Utah Poliomyelitis Research Laboratory; but when unavilable, the tissue nutrient solutions were made from chemically pure reagents.

PROCEDURE FOR PREPARING CHEMICALS

Phosphate Buffered Saline

Phosphate buffered saline (PBS), pH 7.5, was prepared according to the formula:

NaCL	8.0	gm
KCL	0.2	gm
Na_2HPO_4	1.15	gm
KH_2PO_4	0.2	gm
$CaCl_2$	0.1	gm
$MgCL_2.6H_2O$	0.1	gm
H_2O	1000	ml

By omitting $CaCl_2$ this solution was made up 10 times concentrated. Vials of $CaCl_2$ were weighed separately (0.1 gram per vial) for addition to each liter of diluted solution, and sterilized by filtration.

Trypsin solution was prepared from Bacto-Trypsin 1:250. A concentration of 0.25 per cent by weight was prepared using (PBS) as diluent, as prepared by Youngner (32) and was sterilized by Seitz filtration, and refrigerated at 4° C. Prior to use it was warmed to 37° C in a water bath.

Hanks Balanced Salt Solution

Hanks balanced salt solution (BSS) was prepared to a 10 fold concentration, according to the formula:

	GRAMS/LITER
NaCl	80.0
KCL	4.0
$MgSO_4.7H_2O$	2.0
$CaCl_2$	1.4
Na_2HPO_4	0.6
KH_2PO_4	0.6
Glucose	10.0

To each liter of the concentrated stock solution was added 100 ml of 0.2 per cent aqueous solution of phenol red. This mixture was filtered and stored at 4° C. The salt solution used in the cultures was prepared from the stock material in the following way: One part of the concentrated stock was added to 9 parts of resin filtered distilled water. The diluted solution was autoclaved at 10 pounds pressure for 10 minutes. Five tenths ml of an autoclaved 1.4 per cent solution of $NaHCO_3$ was added to each 20 ml of the salt solution just prior to use. The bicarbonate solution was autoclaved separately to avoid the development of a precipitate that occurs if the two solutions are mixed before autoclaving.

Preparation of 1.4 per cent $NaHCO_3$:

One and four-tenths grams of $NaHCO_3$ were dissolved in 100 cc resin filtered water. This solution was dispensed in Erlenmeyer flasks, and autoclaved at 10 pounds for 10 minutes and stored at 4° C with parafilm covers over cotton stoppers. This solution was used, in concentration of 0.5 cc $NaHCO_3$ solution per 20 cc of Hanks solution, to neutralize the latter.

PREPARATION OF MEDIUM D

Balance fluid #199	190 ml	with Phenol red
Horse Serum	40 ml	
$NaHCO_3$ 2.8 per cent	60 ml	with Phenol red
Distilled H_2O	1710 ml	
	2,000 ml	or 2 liters

This solution was sterilized by Seitz filtration.

ANTIBIOTICS ADDED:

Penicillin	100 u/ml
Streptomycin	100 mcg's/ml

Preparation of trypsin-dispersed Cell Suspensions:

Kidneys were removed aseptically from exsanguinated animals. The cortical area was dissected as free as possible from medulla, and then minced with scissors into pieces about 4-5 mm in diameter. The minced cortical tissue was transferred to a 250 ml centrifuge tube, washed several times with (PBS) to remove red blood cells, and finally suspended in about 20 ml of trypsin solution per 5 grams of kidney.

The cells were extracted from fresh tissue by essentially the same method as that described by Youngner (32). Several changes of trypsin, in a concentration of 0.25 per cent diluted in (PBS) and pre-warmed to 37° C, was used in the extractions with the use of a Waring blendor. Each batch of trypsinized cells was filtered through gauze and stored in an ice bath and washed in (BSS) to prevent digestion of cells. The remaining tissue was re-extracted eight times with fresh trypsin.

The turbid trypsinized cell suspension obtained was centrifuged at 1000 r.p.m. for 5 minutes. The sediment was then resuspended in approximately 20 volumes of nutrient solutions (BSS #199 or Hanks) and centrifuged again at 1000 r.p.m. for 5 minutes. Resuspension and washing was repeated for a total of 3 times. After the second washing, the cells were filtered through 3 layers of cheesecloth. The final centrifugation of the pooled cell sediment was performed in a graduated 15 ml centrifuge tube at 600 r.p.m. for 2 minutes.

A yield of from 1.0 to 1.5 ml of packed cellular material was obtained from the cortex of each 5 grams of mouse, monkey, or calf kidney.

Standardization of Cell Suspensions:

The packed cell sediment was diluted with nutrient solution to make a 1:50 dilution based upon packed cell volume. An aliquot of this suspension was further diluted to 1:200 for purposes of standardization. This dilution of the material contained between 600,000 - 700,000 cells per ml.

GLASSWARE PREPARATION

All glassware used in the experiments was boiled in a solution of commercial detergent, rinsed 4-5 times in tap water, soaked for 30 minutes in 0.25 per cent hydrochloric acid, rinsed 5 times in tap water, and then rinsed 4 times in distilled water. The glassware was dried and then autoclaved.

For purposes of continuity, certain other materials and methods utilized will be described with the experimental results.

PART I

ATTEMPTS TO PROPAGATE POLIOVIRUS (Type II MEF-1 Strain) in Mouse Kidney Cells

Virus Propagation:

Attempts to propagate poliovirus (type II MEF-1 Strain) were carried out in mouse kidney fibroblasts, in either suspended cells, or as monolayers in six ounce bottles. Preliminary virus titrations and control tests in monkey kidney cells were regularly run to be sure that the virus was active and that the cell culture system was working properly.

Suspended Cell Technique:

The following suspended cell technique, using trypsinized kidney cells and various virus dilutions, was used:

Virus dilution 0.25 ml, diluting fluid 0.25 ml, and kidney cell dilution 0.25 mlwere added to each Wasserman tube. These tubes were then stoppered with a rubber stopper and incubated at 37° C for 7-10 days. Readings were made beginning the seventh day. All tests were read and analyzed according to the following conditions:

1. An acid color (lemon yellow - Y) indicates growth and active metabolism of the cells, or the absence of

virus.

2. An alkaline color (red PH 7.4 - 7.9 - R) indicates death of the cells, probably due to virus action. Microscopic observations were made to determine the physical state of the cells - if destroyed, there was an absence of cytoplasm and only cell fragments remained. Results of a typical virus titration are presented in Table 1.

VIRUS TITRATION TEST

(suspended monkey kidney cell system)

To each tube was added 0.25 ml of type II mef-1 strain virus dilution, 0.25 ml of diluting fluid, and 0.25 ml of a 1:200 dilution of cell suspension. These tubes were rubber stoppered and incubated 7 - 10 days at 37° C. The fluid system used was medium D, described in "MATERIALS AND METHODS, page 13".

TABLE 1

Virus Dil.	No. Tubes with Virus Growth	No. Tubes No Virus Growth	accumulative		Totals	Per Cent Virus Growth
$10^{-3.5}$	5	0	27	0	27/27	100
10^{-4}	5	0	22	0	22/22	100
$10^{-4.5}$	5	0	17	0	17/17	100
10^{-5}	5	0	12	0	12/12	100
$10^{-5.5}$	4	2	7	2	7/9	78
10^{-6}	2	4	3	6	3/9	33
$10^{-6.5}$	1	4	1	10	1/11	9

Reed and Muench Calculation of the LD_{50} (Am. J. Hyg. Vol: 27, No. 3, 1939)

$$\frac{\text{Per Cent Mortality above 50 - 50}}{\text{Per Cent Mortality above 50 - \% below 50}} \quad \frac{78 - 50}{78 - 33} = \frac{28}{56} = .62$$

The Virus Titer was $10^{-5.62}$.

This titer compares favorably with the titer $10^{-5.5}$ obtained in the Poliomyelitis Laboratory at the University of Utah.

ATTEMPT TO PROPAGATE TYPE II MEF-1 STRAIN POLIOVIRUS IN MOUSE KIDNEY CELLS

To each tube of the test was added 0.25 ml of virus dilution, 0.25 ml of diluting fluid, 0.25 ml of 1:50 through 1:200 dilution of cell suspension. These tubes were rubber stoppered and incubated 7 - 10 days at 37° C. The fluid system used was medium D described in "MATERIALS AND METHODS, page 13".

TABLE 2

Virus Dilutions 0.25 ml	Mouse Kidney Cell Dilutions 0.25 ml 1:50	1:100	1:150	1:200	Control
10^{-1}	Y C	3Y	2Y 1C	3P	1:50 cells 3Y + g
10^{-2}	Y Y	3Y	3Y	2Y 1P	1:100 cells 2Y 1P
10^{-3}	Y C	3Y	2Y 1C	1Y1P1C	1:150 cells 2Y 1P
10^{-4}	Y Y	3Y	3Y	2Y 1P	1:200 cells 1Y 2P + g
10^{-5}	Y C	3Y	3Y	1Y1P1C	
10^{-6}	Y C	3Y	3Y	3P	

Controls: 0.25 ml cell suspension and 0.5 ml diluting fluid.

C - Contaminated, Y - Yellow Acid, P - Pink, g - Good cell layer

Microscopic observation: Cellular growth was observed only in two controls. These tubes were not slanted; this may account for few tubes showing cellular growth microscopically. Pink tubes in the controls must be due to foreign matter toxic to cells.

ATTEMPT TO PROPAGATE TYPE II MEF-1 STRAIN POLIOVIRUS IN MOUSE KIDNEY CELLS

To each tube of the test was added 0.25 ml of virus dilution, 0.25 ml of diluting fluid, and either 0.25 or 0.5 ml of 1:50 through 1:200 dilution of cell suspension. These tubes were rubber stoppered and incubated 7 - 10 days at 37° C. The fluid system used was medium D described in "MATERIALS AND METHODS, page 13".

TABLE 3

Mouse Kidney Cell Dilutions

Virus Dilutions	1:50		1:100		1:150		1:200		Control	
0.25 ml	0.5	0.25	0.5	0.25	0.5	0.25	0.5	0.25	0.5	0.25
10^{-1}	2Y	1Y	1Y1P	1P	1Y1P	1P	2P	1P	1:50 cells 2C	1C
10^{-2}	2Y	1Y	1Y1C	1P	2Y	1P	2P	1P	1:100 cells 2Y + g	1C
10^{-3}	2Y	1Y	2Y	1P	1Y1P	1P	2P	1P	1:150 cells 2Y + g	1Y
10^{-4}	2Y	1Y	2Y	1P	2Y	1P	2P	1P	1:200 cells 1Y1P	1C
10^{-5}	2Y	1Y	1Y 1P	1P	1Y1P	1P	1Y 1P	1P		
10^{-6}	2Y	1Y	2Y	1Y	1Y1P	1Y	1Y 1P	1P		

C - Contaminated, Y - Yellow acid, P - Pink, g - good cell layer

Microscopic observation showed good cellular growth in the Y tubes of greater concentration of cells. The alkaline reaction in tubes in the cell dilution 1:200 could be due to insufficient cells to give the acid reaction, due to growth of the virus, or due to foreign matter toxic to the cells. The tubes of culture showing an alkaline reaction in 1:200 dilution of cells were pooled and frozen to be tested by blind passage technique in monkey kidney cell system and fresh mouse kidney cell system to check possible virus propagation.

Mouse kidney cells were prepared according to the procedure previously described. Typical results, in mouse kidney cells, are presented in Tables 2 and 3. Any tubes suggesting virus growth, (alkaline pink-red pH 7.4 - 7.9), were pooled and second passages were made to other tubes of freshly prepared mouse kidney cells. All positive cultures, suspected of viral propagation, were pooled and frozen for tests on monkey kidney cells. None of these mouse kidney cell virus cultures revealed the presence of viable virus when tested in susceptible monkey kidney cell system. It is concluded, therefore, that poliovirus (type II, MEF-1 strain) failed to propagate in mouse kidney cells under the above experimental conditions.

PART II

ATTEMPTS TO PROPAGATE POLIOVIRUS IN CALF KIDNEY CELLS

These tests were carried out in Melnick's (33) medium consisting of 100 ml of Hanks solution at 10 fold concentration, 5 grams of Lactalbumin hydrolysate, 900 ml of distilled water, 12.5 ml 2.8 per cent $NaHCO_3$, and 20 ml of calf serum. Penicillin 100,000 units and streptomycin 100 mg were added per liter of this medium.

The following virus titrations, using monkey kidney cells and calf kidney cells, were made using Melnick's culture fluid and medium D with (BSS) # 199 for comparison.

Poliovirus types I, Mahoney-strain, type II, MEF-1-strain, type III, Saukett-strain, were obtained from the Poliomyelitis Research Laboratory University of Utah. Pooled virus was made from 3 vials of frozen stock virus for each type.

VIRUS TITRATION IN MONKEY KIDNEY CELLS

TABLE 4

Virus Dilution	Melnick Culture Fluid virus types I	II	III	Medium D Virus Types I	II	III
$10^{-3.5}$	R	R	R	R	R	R
10^{-4}	R	R	R	R	R	R
$10^{-4.5}$	R	R	R	R	R	R
10^{-5}	R	R	R	R	R	R
$10^{-5.5}$	R	R	R	R	R	R
10^{-6}	R	R	R	Y	R	R
$10^{-6.5}$	R	R	R	Y	R	R
10^{-7}	Y	R	R	Y	R	R
Cell Controls No Virus	Y	Y	Y	Y	Y	Y

Color change readings were sharper and more distinct in Melnick's medium than in medium D containing (BSS) #199. Therefore, when solution #199 was no longer available the simpler Melnick medium was used.

VIRUS TITRATION IN CALF KIDNEY CELLS

The calf kidney cells failed to grow in the Wasserman tubes. The same calf cell suspension of 1:200 dilution grew well and produced a good color change (red - yellow) in the next experiment in 6 oz. stoppered bottles.

It was not possible to account for the failure of the calf cells to grow in the Wasserman tubes. These tubes were all slanted during incubation and observed microscopically. No conclusions can be made from this experiment.

BOTTLE TISSUE CULTURE

Monkey kidney cell and calf kidney cell suspensions of 1:200 dilutions were grown in 6 oz. prescription type bottles with rubber stoppers. Melnick's (33) Lactalbumin Hydrolysate calf serum medium was used.

The calf cells were challenged with virus type II, MEF-1 strain, after the third cell transfer showed good monolayer fibroblasts.

Good monolayer fibroblasts were formed in each bottle of monkey kidney cells and also in each bottle of calf kidney cells.

The monkey cells in this experiment were used only for color and pH change comparison and were, therefore, not challenged with virus.

The calf kidney cells were grown in bottles marked I and II. The calf cells in bottle II were infected with a 10^{-3} dilution of stock virus. The cells in bottle I were used for control.

After 7 days, control bottle I still showed a good monolayer of intact fibroblasts, and a good color change.

Viral propagation in bottle II was questionable. However, the fibroblast monolayer did not remain as intact as in Control I. Observation of cell monolayers were made microscopically.

PART III

ATTEMPTS TO PROPAGATE POLIOVIRUS IN CALF KIDNEY CELLS

Calf kidney cells were extracted from freshly obtained kidney by essentially the same method as described in "MATERIALS AND METHODS, page 13". These cells were washed three times and resuspended to a dilution of 1:200 in Melnick's (33) medium consisting of 0.5 per cent lactalbumin enzymatic hydrolysate, 2 per cent calf serum, and 97.5 per cent Hank's salt solution. Penicillin 100,000 units and streptomycin 100 mg were added per liter.

Monolayer fibroblasts were grown in bottles and the nutrient fluids changed every 3 - 4 days. After the microscopic examination revealed a good monolayer of fibroblasts these cells were again trypsinized and resuspended in Melnick's medium. Some of these cells were used in Wasserman tubes with Type II, MEF-1 strain virus, and the remainder of the cells were transferred to another bottle for a second generation. This cell transfer was carried out for 10 generations. (See Table 7)

The first monolayer of cells was used for Series "A" Wasserman tube tests. The second generation monolayer was trypsinized and divided into "A" Series and "B" Series Wasserman tubes, and the remainder seeded to another bottle for the third generation. The "A" Series tubes followed a blind passage of virus from original Wasserman tube virus to second generation "A" Series, to third generation "A" Series etc. The "B" Series of the second generation of cells was seeded with fresh virus suspension 10^{-3} and carried by blind passage into the third generation "B" Series etc.

The first, third, sixth and ninth blind passage of series "A", "B", "C", etc., were pooled and stored in the deep freeze to be tested in monkey kidney cells for any possible virus propagation.

Fresh poliovirus (Type II), strain MEF-1 was used in all new series (eg. A 1, B 1, C 1, etc.) tests and in monkey kidney cell controls. (See Table 7)

Fresh calf kidney cell suspensions were made as mentioned above and dispensed in sterile bottles for the first cell generation. These bottles were stoppered with rubber stoppers.

Amount of 1:200 cell suspension	Bottle size	Number of Bottles
50 ml per bottle	16 oz.	6
75 ml per bottle	1 liter bottles	2
25 ml per bottle	8 oz.	2

Microscopic examination revealed good monolayer fibroblasts. The 16 oz. bottles, used to grow the calf cells, were numbered I through VI.

The first generation monolayer cells, in bottles I and II were trypsinized. These cells were centrifuged and washed three times and then resuspended in 1:200 dilution of Melnick's fluid.

Bottles	Approximate Cell Yield
I	0.1 ml packed cells
II	0.2 ml packed cells

The cells from number I bottle were resuspended in bottle number I 2 for a second generation.

The cells from number II bottle were resuspended and dispensed in new bottles for a second generation:

Bottles	
II 2	16 oz.
II 2A	8 oz.
II 2B	8 oz.

Each of the following cell passage bottles, 16 oz. and 8 oz., received 20 ml and 5 ml respectively of a 1:200 dilution of cells; and the remainder of each generation passage cell suspensions were used in the blind passage Series tests.

BLIND PASSAGE SERIES A 1

Each tube (1 - 8) contained 0.25 ml 1:200 calf kidney cells, 0.25 ml Melnick's diluting fluid, and 0.25 ml fresh virus diluted in Melnick's nutrient fluid to 10^{-3}.

Each tube (9 - 12) contained 0.25 ml 1:200 calf cells, and 0.5 ml virus suspension.

Each control tube (13 - 14) contained 0.5 ml 1:200 calf cells, and 0.25 ml diluting fluid.

Tube Numbers	1	2	3	4	5	6	7	8	9	10	11	12	13	14
	M	O	M	M	M	M	M	M	M	M	O	M	M	M

M - good monolayer with acid yellow

O - degenerated monolayer yellow-orange

A good cell layer of calf kidney fibroblasts was produced in controls as well as all tests except tubes number 2 and 11. These two tubes were pooled and blind passaged in calf kidney cells trypsinized from bottle II 2A second generation. Part of pooled tubes number 2 and 11 was stored in the deep freeze to be tested later in monkey kidney cells to see if possible cell degeneration was due to virus propagation.

BLIND PASSAGE SERIES A 2

Each tube (1 - 2) contained 0.25 ml fluid pooled from tubes number 2 and 11 of Wasserman (Series A 1) tubes, 0.25 ml diluting fluid, 0.25 ml calf kidney cells in 1:200 dilution from second generation bottle II 2.

Test Tube Number	1	2
	M	M

M - Good cell layer with acid was formed.

No viral propagation was indicated.

After microscopic observation revealed a good solid monolayer bottle II 2A was seeded with 30 ml of Fresh virus diluted to 10^{-3}. Some areas showed fibroblast degeneration after 6 days. The clear fluid and concentrated cells from bottle II 2A were sealed in separate glass ampules. These ampules were kept in the deep freeze for testing later in monkey kidney cells to see if any virus propagation took place; this may be noted by any rise in titer.

Calf cells in bottle II 2 were trypsinized and resuspended in Melnick's nutrient fluid and dispensed in new bottles for a third generation:

<u>BOTTLES</u>

II 3 16 oz.

II 3A 8 oz.

II 3B 8 oz.

The remainder of these cells were used in Wasserman tube tests Series B1 calf cells with fresh 10^{-3} diluted virus added.

Each tube contained 0.25 ml II 2 cells, 0.25 ml Virus dilution, and 0.25 ml Melnick's diluting fluid.

Tube Number	1	2	3	4	5	(6 control)
	M	M	M	M	M	M

Good monolayer fibroblasts were formed and all tubes turned yellow acid.

A fourth generation ofcalf kidney cells was started by trypsin digestion of the cell monolayer in bottle II 3A. These cells were washed and resuspended in Melnick's solution containing 2 per cent calf serum. This suspension of cells in a 1:200 dilution was dispensed in new bottles for a fourth generation:

<u>BOTTLES</u>

II 4 16 oz.

II 4A 8 oz.

II 4B 8 oz.

The remaining 10 ml of this cell suspension was used for Wasserman tube tests. Suspected virus for the series Blind Passage Tests was used from preceding Blind Passage Tests. Each new series eg. A 1, B 1, C 1, etc., was started with fresh virus.

BLIND PASSAGES SERIES A 3, B 2, C 1

<u>Series A 3:</u>

Tube Number	<u>1</u>	<u>2</u>	<u>3</u>	<u>4</u>	(<u>5 Control</u>)
	M	M	M	M	M

No evidence of virus propagation was noted. The solution from tubes 1 - 4 was pooled and frozen in 2 vials to be tested later in monkey kidney cells.

<u>Series B 2:</u>

Tube Number	<u>1</u>	<u>2</u>	<u>3</u>	<u>4</u>	<u>5</u>	<u>6</u>	(<u>7Control</u>)
	M	M	M	M	M	M	M

A good cell layer formed in each tube and all tubes were yellow acid. There was no evidence of virus propagation.

Fresh virus dilution 10^{-3} was used in Series C 1

<u>Series C 1:</u>

Tube Number	<u>1</u>	<u>2</u>	<u>3</u>	<u>4</u>	(<u>5 Control</u>)
	M	M	M	M	M

Fair cell layers were formed. Some cells were rounded. The solution from tubes 1 - 4 was pooled and saved in two vials for testing in monkey kidney cells.

On 3-7-56 the small 8 oz. bottle I, of original cells, was circled over areas of good solid monolayers and these areas were spotted with type II, MEF-1 strain, virus diluted 10^{-3} using a sterile loop. After 6 days these areas showed some degenerated cells. Material from these areas was blind passaged 3-14-56 to two tubes containing calf kidney cells. Blind passaged from these tubes to new tubes containing calf kidney cells 3-21-56. These two tubes showed 1 pink with no cell growth and 1 orange tube with no cell growth. Solutions from these two tubes were used in seperate Blind passage tests 1 and 2, using fresh calf kidney cells.

1 Pink Tube	Tube Number	1	2	(3 Control)
		M	M	M
2 Orange Tubes	Tube Number	1	2	(3 Control)
		M	M	M

Good cell layers were formed and the carbonic acid formed by cellular metabolism turned the indicator system yellow in all tubes. Virus did not propagate in these tests.

The cell monolayer in 8 oz. bottle II 3A was challenged with fresh virus (20 ml) diluted to 10^{-3}.

Some cell degeneration was noted. However, in the control (II 3B) most of the cells had fallen off. Therefore, II 3A results are not significant. Three ampules were made and frozen from supernate of II 3A to be tested later in monkey cells.

Cells in bottle II 4 showed a good monolayer. This monolayer was trypsinized, washed 3 times, resuspended in Melnick's solution, and then dispensed in new bottles for a fifth generation:

BOTTLES	
II 5	16 oz.
II 5A	8 oz.
II 5B	8 oz.

BLIND PASSAGE SERIES A 4

Each tube contained 0.25 ml suspected virus blind passage, 0.25 ml diluting fluid, and 0.25 ml 1:200 calf kidney cells from bottle II 4.

Series A 4, B 3, C 2, D 1:	Tube Number				
	1	2	3	4	(5Control)
A 4 (from pooled A 3)	M	M	M	M	M
B 3 (from pooled B 2)	M	M	M	M	M
C 2 (from pooled C 1)	M	M	M	M	M
D 1	M	M	M	M	M

Fresh poliovirus suspension 10^{-3} was added to D 1.

All tubes, tests as well as controls, showed good cell layer fibroblast formation although color or pH change showed a slight pink in all tubes. Each cell layer seems to have more rounded cells than the first few passages; however, the majority of the fibroblasts are still elongated.

Five ml of a 1:200 dilution of calf kidney cells from bottle II 4 was placed in each of 4 petri plates and placed in a dessicator (candle lit before sealing), to grow monolayers for plaque method (34). The fluids were changed after noting scattered colonies of calf kidney cell fibroblasts that were attached to the petri plates. This monolayer formation was not successful. Degeneration had taken place. Therefore, these plates were not used for plaque method. The explanation for failure of the monolayer to grow in petri dishes may be the fact of using ordinary petri dishes and also failure to maintain proper CO_2 balance.

The cell monolayer in small bottle II 4A was challenged with fresh virus diluted to 10^{-3} (20 ml). Complete degeneration of these cells was noted after 3 days. Fluids from this bottle were saved in 3 ampules and frozen in the deep freeze, to be tested later in monkey kidney cells.

Control bottle II 4B monolayer also showed considerable cell degeneration such that bottle II 4A test with virus may not be significant.

Difficulty encountered in growing calf kidney cell monolayers in the small 8 oz. bottles could be due to greater demand for cells in the Wasserman blind passage tests and therefore using too small an inoculum of cells. These monolayers seemed to progress for the first 7 days but after 10 days or longer the controls (with addition of fresh nutrients every third and forth day) also degenerated. It was not possible to account for this difficulty unless the small bottles were made of a different type of glass that could have been detrimental to maintenance of cell monolayers. This difficulty was not encountered in the larger bottles.

Good monolayers were formed in the fifth generation cell passage, although some areas of the monolayer were not completely filled in with cells. More cells were rounded as compared to cells in the first generation; however, the greater majority of cells were elongated fibroblasts.

The cell monolayer in bottle II 5 was trypsinized and the cells washed 3 times in Melnick's medium, resuspended and dispensed in new bottles, for a sixth generation, then stoppered and the results read after 7 days at 37° C.

BOTTLES		RESULTS
II 6	16 oz.	Good monolayer
II 6A	8 oz.	Degenerated cell forms shriveled possibly due to something in bottle toxic to cells.
II 6B	8 oz.	Cells seem to be more rounded yet elongated.

A new batch of Melnick's medium was used in bottle II 6B. The remainder of these cells were used in the Wasserman tube blind passage test Series A 5. Each series received 0.25 ml of suspected virus from the previous blind passage tests.

Example: A 5 received 0.25 ml in each tube from series A 4 etc.

BLIND PASSAGE SERIES A 5

Series A 5, B 4, C 3, D 2, E 1:

	Tube Number 1	2	3	4	5	6	(7 Control)
A 5	M	M	M	M	M	M	M
B 4	M	M	M	M	M	M	M
C 3	M	M	M	M	M	M	M
	Pooled for testing in Monkey cells						
D 2	M	M	M	M	M	M	M
E 1	M	M	M	M	M	M	M

Fresh virus 10^{-3} from vial No. 2 was used in Series E 1.

Series E 1 solutions were pooled for testing in monkey cells.

The pH change was excellent in all tubes of this series. The red-yellow change indicates that the calf kidney cells can be utilized in the system for testing possible virus adaptation.

MID-WAY TESTS IN MONKEY KIDNEY CELLS

(Of suspected virus from calf kidney cell blind passage tests)

Each tube contains 0.25 ml of test solutions in each dilution respectively, (3 tubes each dilution); 0.25 ml diluting fluid, and 0.25 ml of a 1:200 dilution of monkey kidney cells received from Poliomyelites virus research Laboratory, University of Utah, 4-4-56, 5 P.M. and refrigerated 1:50 overnight. These cells were diluted to 1:200 for the tests.

VIRUS DILUTION PROCEDURE

0.25 ml virus	→ 0.25	→ 0.25	→ 0.25	
Dil. Fluid	2.25	2.25	2.25	2.25
Undilute	10^{-1}	10^{-2}	10^{-3}	10^{-4}

TABLE 5

(SUSPECTED VIRUS TESTED IN MONKEY KIDNEY CELL SUSPENSIONS)

		VIRUS DILUTIONS					
Sample #	Series #	Undiluted	10^{-1}	10^{-2}	10^{-3}	10^{-4}	(Control
I	A 1	1 or. red	3M	3M	3M	3M	3M
II	II 2B	3 or. red	3Y	3M	3M	3M	3M
III	II 2B	2 red	3Y	3M	3M	3M	3M
IV	A 3	3M	3M	3M	3M	3M	3M
V	C 1	3Y	3M	3M	3M	3M	3M
VI	I 8oz.	3M	3M	3M	3M	3M	3M
VII	I 8oz.	3M	3M	3M	3M	3M	3M
VIII	II 3A	2Y	3M	3M	3M	3M	3M
IX	II 4A	1Y	3M	3M	3M	3M	3M
	Fresh virus dilutions from #3 vial was used in the Control.						
X	Control	3R	3R	3R	3R	3Or.red3Y	

R Tubes - No cell monolayers

Y Tubes - Degenerated monolayer

M Tubes - Good monolayers

The virus titer in the control is approximately 10^{-4}.

The test solutions of Series numbers II 3A, II 2B, etc., are from the cell monolayers that were challenged with virus type II in bottles. The test solutions of numbers C 1, A 3, etc., are from the pooled virus blind passage Wasserman tube tests.

The cell monolayer in 16 oz. bottle II 6 was trypsinized, washed 3 times and resuspended in Melnick's solution, and then dispensed in new bottles for a seventh generation.

BOTTLES	
II 7	16 oz.
II 7A	8 oz.
II 7B	8 oz.

The remaining 10 ml was used in Wasserman tube tests.

BLIND PASSAGE SERIES A 6

Each tube contains 0.25 ml from previous blind passage ie. A 5 - A 6 etc., 0.25 1:200 Calf Cells, and 0.25 Melnick dilution fluid.

Series A 6, B 5, C 4, D 3, E 2, F 1:

	Tube Numbers						
	1	2	3	4	5	6	(Control)
A 6	M	M	M	M	M	M	M
B 5	M	M	M	M	M	M	M
C 4	M	M	M	M	M	M	M
D 3	M	M	M	M	M	M	M
E 2	M	M	M	M	M	M	M
	Fresh virus 10^{-3} dilution from #3 vial was used.						
F 1	M	M	M	M	M	M	M

All tube solutions were more orange red in comparison to yellow tubes in the previous Blind Passage Series A 5. However, good monolayers formed in all tubes. These cells were more pleomorphic and elongated.

The monolayer in 8 oz. bottle II 5A which was challenged with type II poliovirus, showed more degenerated cells than control II 5B although II 5B also showed some rounding and degeneration. Three ampules were made of supernatant fluids in bottle II 5A to be tested later in monkey kidney cells.

The calf cell monolayer in 8 oz. bottle II 6B was challenged with fresh virus 20 ml of 10^{-3} dilution. The cells in control bottle II 6A showed degenerated and shriveled forms, possibly something in the bottle was toxic to this cell monolayer. The cells in test bottle II 6B all shriveled up. Some of the solution from bottle II 6B was saved in ampules.

The calf cell monolayer in 16 oz. bottle II 7 was trypsinized, washed 3 times, resuspended in 40 ml Melnick's solution, and distributed to new bottles for the eighth generation.

BOTTLES		RESULTS
II 8	16 oz.	Good monolayer pleomorphic.
II 8A	8 oz.	Sparce monolayer.
II 8B	8 oz.	Fair monolayer.

The remainder of these cells were used in the following Wasserman tube tests.

BLIND PASSAGE SERIES A 7

Series A 7, B 6, C 5, D 4, E 3, F 2, G 1:

Tube Number

	1	2	3	4	5	6	(Control)
A 7	M	M	M	M	M	M	M

	Tube Number Continued						
	1	2	3	4	5	6	(Control)
B 6	M	M	M	M	M	M	M
C 5	M	M	M	M	M	M	M
D 4	M	M	M	M	M	M	M
E 3	M	M	M	M	M	M	M
F 2	M	M	M	M	M	M	M
	Fresh virus 10^{-3} dilution was used in series G 1.						
G 1	M	M	M	M	M	M	M

All tubes were more yellow than the orange red of the last Blind Passage Series A 6 test. The cells were elongated and somewhat more rounded.

The monolayer in bottle II 7B was challenged with fresh virus diluted 10^{-3}. A very good monolayer was maintained for 7 days after 25 ml of a 10^{-3} dilution of poliovirus type II MEF-1 strain was added. Actually the monolayer in the test bottle was better than the monolayer in bottle II 7A that was used as the control. The virus did not induce any visable damage to the cells in this test.

The calf cell monolayer in the original 16 oz. bottle III 1, that had fluids changed each third and fourth day for two months, was trypsinized and washed 3 times in Melnick's to see if the monolayer cells were still viable. These cells were resuspended in bottle III 2. A good complete monolayer was formed after 6 days growth. The original bottles of cells have been maintained in a viable condition. Some of these cells were used in the following Wasserman tube test challenged with fresh diluted 10^{-3} virus.

Series:

	Tube Number						
	1	2	3	4	5	6	(4 Controls) no virus
III 1	M	M	M	M	M	M	4M

All solutions in the tubes were yellow after 10 days and showed good cell layers. The cell layers from III 1 are not as elongated nor as clearly nucleated as Blind Passage Series tests using cells from the seventh generation.

The calf serum concentration was increased to 4 per cent in Melnick's medium and used in the subsequent tests to try and overcome the thin elongation of calf kidney cells observed in previous Blind Passage Series tests.

The monolayers in 16 oz. bottle II 8 and 8 oz. bottle II 8B were trypsinized and combined and seeded into 16 oz. bottle II 9 for the ninth generation. The eighth generation monolayer in stock bottle II 8 was not a solid monolayer. This could be attributed to a greater demand on cells to run the Blind Passage Series tests. Therefore, small 8 oz. bottles were not used for subsequent monolayers.

Combined cells from bottles II 8 and II 8B were also used in Wasserman Tube Blind Passage Series.

BLIND PASSAGE SERIES A 8

Each tube contained 0.25 ml suspected virus suspension, 0.25 ml of a 1:200 dilution of calf kidney cells, and 0.25 ml Melnick's diluting fluid.

Series: A 8, B 7, C 6, D 5, E 4, F 3, G 2, H 1:

	Tube Number						
	1	2	3	4	5	6	(No virus Control)
A 8	M	M	M	M	M	M	M
B 7	M	M	M	M	M	M	M
C 6	M	M	M	M	M	M	M
D 5	M	M	M	M	M	M	M
E 4	M	M	M	M	M	M	M
F 3	M	M	M	M	M	M	M
G 2	M	M	M	M	M	M	M

Fresh virus diluted 10^{-3} was used in Series H 1.

H 1	M	M	M	M	M	M	M

The calf cells in this series were more rounded than the thin elongated cells in the previous Blind Passage Series tests that contained only 2 per cent calf serum.

Conclusion: The use of 4 per cent calf serum in Melnick's medium aids the growth of the cells.

FINAL TESTS IN MONKEY KIDNEY CELLS

(Of suspected virus from calf kidney cell Blind Passage Tests)

Monkey kidney cells were obtained from the Poliomyelitis Virus Laboratory, University of Utah. These cells were refrigerated overnight and diluted to a 1:200 final suspension. This suspension of cells was used to test possible viral propagation in calf cell Blind Passage Series tests D 1 through B 6.

Suspected virus dilutions were made following the same procedure listed on page 41. Three tubes were utilized for each dilution. Each tube contained 0.25 ml of suspected virus solution undiluted, 10^{-1} through 10^{-4}, 0.25 ml of diluting fluid, and 0.25 ml of a 1:200 dilution of monkey kidney cells. (See Table 6)

TABLE 6

Series	Virus Dilutions Undilute	10^{-1}	10^{-2}	10^{-3}	10^{-4}	(Control)
D 1	3R	3R	3Y	3Y	3Y	3Y
B 3	3Y	3Y	3Y	3Y	3Y	3Y
E 1	3R	1R 2Y	3Y	3Y	3Y	3Y
C 3	3Y	3Y	3Y	3Y	3Y	3Y
Bottle II 5A	3R	2Y 1R	3Y	3Y	3Y	3Y
F 1	3Y Degenerated	3Y Rounded Shriveled	3Y (- - - good cell layers - - -)	3Y	3Y	3Y
D 3	3Y	3Y	3Y	3Y	3Y	3Y
A 6	3Y	3Y	3Y	3Y	3Y	3Y
G 1	3R	3Y	3Y	3Y	3Y	3Y
E 3	3Y	3Y	3Y	3Y	3Y	3Y
B 6	3Y	3Y	3Y	3Y	3Y	3Y
	Fresh thawed virus from #3 vial was used in Control Test.					
Virus Control	3R	3R	3R	3R	3R	3Y

R - degenerated cells - virus propagation

Y - good cell layer

The titer of the virus control is at least 10^{-4}, and all these red tubes show degeneration and disintegration of cells. The controls showed good cell layers.

It is of interest to note in each series where fresh virus had been added to calf kidney cells and pooled after 7 days incubation, that this pooled suspected virus titer was approximately 10^{-1} when tested in monkey kidney cells.

The calf cell monolayer in 16 oz. bottle II 9 was trypsinized and washed three times and resuspended in Melnick's 4 per cent calf serum medium. These cells were distributed to 16 oz. bottle II 10 for the tenth generation, and the remainder of these cells were used in Blind Passage Series A 9.

BLIND PASSAGE SERIES A 9

Series A 9, B 8, C 7, D 6, E 5, F 4, G 3, H 2, I 1:

	Tube Number					
	1	2	3	4	5	(Control)
A 9	M	M	M	M	M	M
B 8	M	M	M	M	M	M
C 7	M	M	M	M	M	M
D 6	M	M	M	M	M	M
E 5	M	M	M	M	M	M
F 4	M	M	M	M	M	M

	Tube Number Continued					
	1	2	3	4	5	(Control)
G 3	M	M	M	M	M	M
H 2	M	M	M	M	M	M

Fresh virus diluted 10^{-3} was used in series I 1.

I 1	M	M	M	M	M	M

There was no apparent virus propagation. However, these calf cells were more rounded and yet they were still elongated after 10 generation passages.

SYSTEM USED IN ATTEMPTS TO PROPAGATE POLIOVIRUS IN CALF KIDNEY CELLS

Calf kidney cells were extracted from fresh tissue as described in "MATERIALS AND METHODS page 13". These cells were suspended in Melnick's medium and grown in bottles for the first generation of cells. These cells were grown for ten consecutive generations. Some calf kidney cells from each new generation were used in Wasserman tube Blind Passage Series tests. The first generation of cells was divided; some of these cells were used as subplants for a second generation cell passage. The remainder of these cells were used in Series A 1

Wasserman tubes and infected with fresh virus type II, MEF-1 strain. The second generation of cells were divided; some of these cells were used as subplants for a third generation cell passage. The remainder of these cells were used in Series B 1 and A 2 Wasserman tubes. In Series B 1, these cells were infected with fresh virus. In Series A 2, these cells were infected with material from Series A 1 by blind passage. This general pattern was carried out for ten generations. (See Table 7)

TABLE NO. 7

Cell Generation	Wasserman Tube Cell Culture Series
1	A 1
2	A 2, B 1
3	A 3, B 2, C 1
4	A 4, B 3, C 2, D 1
5	A 5, B 4, C 3, D 2, E 1
6	A 6, B 5, C 4, D 3, E 2, F 1
7	A 7, B 6, C 5, D 4, E 3, F 2, G 1
8	A 8, B 7, C 6, D 5, E 4, F 3, G 2, H 1
9	A 9, B 8, C 7, D 6, E 5, F 4, G 3, H 2, I 1
10	A10, B 9, C 8, D 7, E 6, F 5, G 4, H 3, I 2

I - Cells from generation 1 were transferred to generation 2 - to 3 - to 4 etc. For 10 generations without any viral infection.

II - Each new series A 1, B 1, C 1, D 1, etc., consisted of cells from generation 1, 2, 3, 4, respectively, and was mixed with fresh virus diluted to 10^{-3}. Each Blind Passage Series A 2, A 3, etc., B 2, B 3, etc. was infected with material from previous series (eg. A 2 from A 1).

III - Fluids, from Wasserman tube Series A 1, A 3, A 6, A 9; B 1, B 3, B 6, B 9, etc., from 10 generation calf kidney cells Blind Passage tests, were tested in monkey kidney cells to determine whether or not the virus actually propagated in calf kidney cells. Any propagation of virus would be noted by a rise in titer above the dilution 10^{-3} of virus used in these tests.

DICUSSION

Mouse as well as calf kidney cells, grown in (BSS) containing phenol red indicator turned the solutions from red to yellow acid within seven days. This indicated that these cells not only metabolized but propagated and may be used for testing any possible viral multiplication by the same system used for testing viral propagation in monkey kidney cells.

In these experiments, using mouse and calf kidney cells, it was found best to slant the tubes during incubation in order to facilitate microscopic examination of the cell layers.

Virus titrations, using monkey and calf kidney cells, were run using Melnick's culture fluid (33) and medium D with (BSS) 199 for comparison. It was found that the virus titration tests were sharper and more distinct using Melnick's medium. Therefore, after solution #199 was no longer available the simpler Melnick medium, consisting of calf serum and Lactalbumin Hydrolysate in Hanks (BSS), was used. Melnick's nutrient fluids were used in Part II and Part III of this experiment.

Calf kidney cells were used in Part II and

Part III to see if these cells could support the growth of poliovirus and thus account for the presence of specific neutralizing antibodies in cows. If this were possible it would also confirm the hypothesis of Bartell and Klein (30) that cows could be an extra-human reservoir of the poliovirus.

Calf kidney cells, in Part III, were trypsinized and transferred successfully for 10 generations. Such cells that have been thoroughly adapted to _in vitro_ multiplication according to Smith, _et al_, (19) often change their biological characteristics. It appeared desirable to determine whether these calf kidney cells, under the stress of continued new generations and through Viral Blind Passage Tests, could support the growth of poliovirus.

Although no actual viral propagation was indicated in either mouse or calf kidney cells, by the method employed, it is possible that only a very small number of cells in any given population would be susceptible to the virus (26). Any occurrence of a very small number of susceptible cells in a given population (eg. 60 cells in a population of 10^5 cells being infected) according to Kaplan and Melnick (26) would result in a failure to observe any cytologic changes. It is possible that small numbers of cells, mouse as well as calf kidney, could have supported the growth of the poliovirus and gone undetected by the tissue culture tube method employed.

Solutions from each new calf kidney cell series that was pooled one week after challenge with fresh type II, MEF-1 strain poliovirus titrated 10^{-1} in monkey kidney cells. This titer compares to the titer of virus pooled after one weeks growth in tube cultures of monkey kidney cells. The virus titer, of the solution pooled from monkey cells, was too low to be used as stock virus. This indicates that it is probably due to a virus carry over. However, it is possible that a few calf kidney cells, susceptible to the virus, could account for these cells maintaining the virus titer comparable to the titer maintained by monkey kidney cells.

Further tests with calf kidney cells as well as other non-primate tissue cells should be made using Dulbeccos plaque method (34) to determine more critically whether or not these cells are susceptible to the polioviruses.

SUMMARY

The propagation of poliovirus was not indicated in either mouse or calf kidney cells by the method employed.

The titer of poliovirus, that has been incubated one week in tubes of calf kidney cells, compares to the titer (10^{-1}) of poliovirus incubated one week in tubes of monkey kidney cells. This low titer is probably due to inactivation of the virus by prolonged incubation, or due to some weakness in the suspended cell tube system.

The titer, of poliovirus that propagated in monkey kidney cells after incubation for one week, was too low for this source of virus to be used as stock virus.

The Melnick (33) end point, using calf serum Lactalbumin hydrolysate in Hanks (BSS) was sharper and more distinct than medium D with (BSS) # 199. The simpler method by Melnick was used in the majority of these tests.

The use of Melnick's medium containing only 2 per cent calf serum resulted in thin elongation of the calf kidney cells after the sixth generation passage.

Increasing the serum content in this medium to 4 per cent aided the growth of the cells. The calf kidney cells grown in Melnick's medium containing 4 per cent calf serum were more rounded yet still elongated after ten generations.

In order to facilitate microscopic examination of cell layers in tube cultures it was necessary to slant the tubes during incubation.

REFERENCES

(1) Landsteiner, K and Popper, E., Ubertragung der poliomyelitis acute auf affen ztschr. F. Immunitatsforsch. U. Exper. Therap., 2:377, 1909.

(2) Enders, J.F. Weller, T.H., and Robbins, F.C., Cultivation of the lansing strain of poliomyelitis virus in cultures of various human embryonic tissues. Sc. 109:85. 1949.

(3) Rhodes, A.J. et al, Cultivation of poliomyelitis virus in tissue culture. VI. Methods for quantity production of poliomyelitis viruses in cultures of monkey kidney. Canadian J. Pub. Health, 44:273, 1953.

(4) Syverton J.T., Scherer W.F., Elwood P.M., Studies on the propagation in vitro of poliomyelitis viruses. V. The application of strain He La human epithelial cells for isolation and typing. Jour. Lab. Clin. Med. 43:2, Feb. 1954.

(5) Enders, J.F., General preface to studies on the cultivation of poliomyelitis virus in tissue culture. J. of Imm. 69:639, 1952.

(6) Salk, J.E. Youngner J.S. et al, Antigenic activity of poliomyelitis vaccines undergoing field test. Am. J. Pub. Health 45:151, 1955.

(7) Salk, J.E. Studies in human subjects on active immunization against poliomyelitis, J. A. M. A. 151:1081, 1953.

(8) Sabin, A.A., Present status and future possibilities of vaccine for control of poliomyelitis. A. M. A. Am. J. Dis Child. 86:301, 1953.

(9) Bodian, D., Concepts and problems of immunization against poliomyelitis. Fed. Proc. Baltimore, 13:685, 1954.

(10) Cox, H.R. Living modified viruses as immunizing agents Brit. M. J. 2:no. 4882, 259-265, 1954.

(11) Kaprowski, H. et al, Immunization of man with living poliomyelitis virus. W. H. O. Monograph Series No. 26:335-356, 1956.

(12) Kaprowski, H. et al, Clinical investigations on attenuated strains of poliomyelitis virus. J. A. M. A. 160: 954, 1956.

(13) Salk, J.E., Present status of the problem of vaccination against poliomyelitis. Am. J. Pub. Health 45:285, 1955.

(14) Scheele, L. A., and Shannon, J. A., Public health implications in a program of vaccination against poliomyelitis. J. A. M. A. 158:1249, 1955.

(15) Peterson, L. J. et al, Vaccination-induced poliomyelitis in Idaho-Preliminary report of experience with Salk poliomyelitis vaccine. Ibid. 159:241, 1955.

(16) Salk, J. E., Poliomyelitis vaccine in the fall of 1955. Am. J. Pub. Health 46:1, 1956.

(17) Weller T. H., Enders, J. F., et al, Studies on the cultivation of poliomyelitis viruses in tissue culture. J. Imm. 69:645, 1952.

(18) Robbins F. C., Enders, J. F., et al, Studies on the cultivation of poliomyelitis viruses in tissue culture. J. Imm. 69:673, 1952.

(19) Smith W. M., Evans C. A., et al, Growth of neurotropic viruses in extraneural tissues. V. Survey of tissues of various animal species for capacity to support multiplication of poliomyelitis virus in vitro. J. Infect. Dis. 94:3, 1954.

(20) Kast C., Kolmer J. A., Unsuccessful attempts to cultivate the virus of epidemic poliomyelitis in various living tissue mediums. J. Infect. Dis. 61:60, 1937.

(21) Original not seen; cited in ref. (20) Med. Jour. Australia 1:36, 1955.

(22) Garcia R., et al, Poliomyelitis II. propagation of MEF-1 strain of poliomyelitis virus in developing chick embryo by yolk sac inoculation. Proc. Soc. Exper. Biol & Med. 81:519, 1952.

(23) Cabasso, V. J., Cox, H. R., et al, Poliomyelitis IV some cultural and other characteristics of chick-embryo-adapted type II strain of poliomyelitis virus. Proc. Soc. Exp. Biol. N. Y. 85:1 167-71, 1954.

(24) Melnick, J. L., and Paul, J. R., Susceptibility of cebus capucina (So. Am. Ringtail Monkey) to poliomyelitis virus. J. Exp. Med., 78:273, 1943.

(25) Kaplan, A. S., The susceptibility of kidney cells to poliovirus in vivo and in vitro. Virology 1:377, 1955.

(26) Kaplan, A. S., and Melnick, J. L., Multiplication of virulent poliovirus in capuchin monkey cultures without microscopically observed cytopathogenicity. Proc. Soc. Exp. Biol. and Med. 90:562, 1955.

(27) Hammon, W. Med., et al, The significance of protection tests with the serum of man and other animals against the lansing strain of poliomyelitis virus. J. Imm. 57:285, 1947.

(28) Gordon, F. B., The neutralization of poliomyelitis virus by dog serum., J. Inf. Dis., 76:198, 1945.

(29) Grear, J. S. H., Immunity to poliomyelitis. Ann. Int. Med. 37:1, 1952.

(30) Bartell, P., and Klein M., Neutralizing antibody to viruses of poliomyelitis in sera of domestic animals. Proc. Soc. Exp. Biol. and Med. 90:597, 1955.

(31) Miller, C. A. and Wenner, H. A., Neutralization of poliomyelitis viruses by serums from apparently normal monkeys. Am. J. Hyg. 58:47-51, 1953.

(32) Youngner, J. S. Monolayer tissue cultures. I. Preparation and standardization of suspensions of trypsin-dispersed monkey kidney cells. Proc. Soc. Exptl. Biol. Med. 85:202-205, 1954.

(33) Melnick, J. L. et al, Stabilized suspensions of monkey kidney cells suitable for intercontinental shipment. Proc. Soc. Exper. Biol. Med. Vol. 88:676, 1955.

(34) Dulbecco R., Vogt. M. Plaque formation and isolation of pure lines with poliomyelitis virus. J. Exptl. Med. 99:167-132, 1954.

THE FEASIBILITY OF PROPAGATING POLIOVIRUS IN NON-PRIMATE TISSUES

by

E. ROYLANCE MARTIN

An abstract of a thesis submitted
to the faculty of the University
of Utah in partial fulfillment of
the requirements for the degree of

MASTER OF ARTS

Approved by the faculty committee in

August, 1956

LOUIS P. GEBHARDT, CHAIRMAN, SUPERVISORY COMMITTEE

Department of Bacteriology

University of Utah

August, 1956

Tests concerning the feasibility of propagating poliovirus in non-primate tissues were conducted over a period of two years. The purpose of these tests was to determine whether the cellular specificity of polioviruses was limited to cultures of primate tissues.

Mouse and calf kidney cells were grown in bottles and transferred for several generations. Cells from each new generation were challenged with type II MEF-1 strain poliovirus. The monkey kidney cell system was used for controls.

The use of Melnick's medium containing only 2 per cent calf serum resulted in thin elongation of the calf kidney cells after the sixth generation passage. Increasing the serum content in this medium to 4 per cent aided the growth of the cells. The calf kidney cells grown in Melnick's medium containing 4 per cent calf serum were more rounded yet still elongated after ten generations.

The propagation of poliovirus was not detected in repeated experiments with freshly prepared as well as several subculture generations of either mouse or calf kidney cells by the method employed. It is possible as in the case of Capuchin monkey kidney cells that only a very small number of cells in any given population would be susceptible to the virus. Any occurrence of a very small number of susceptible cells in a given population would result in a failure to observe any cytologic changes. It is possible that small numbers of cells, mouse as well as calf kidney, could have supported the growth of the poliovirus and gone undetected by the tissue culture tube method employed.